

## SUMMARY

The C<sub>17</sub> branched-chain fatty acid 15-methylhexadecanoic acid has been isolated in trace quantities (approximately 0.06 % of total fatty acids) from hydrogenated ox perinephric fat.

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## The Composition of Collagen and Acid-Soluble Collagen of Bovine Skin

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The partial dissolution of collagen in dilute solutions of weak acids, such as formic and acetic, has been known for some years, and has been extensively studied by Nageotte (1927*a, b*, 1928, 1930, 1933), Nageotte & Guyon (1933), Leplat (1933), and Fauré-Fremiet (1933). Interest in this soluble protein has been revived in recent years by the work of Orekhovich and his colleagues in the U.S.S.R., who report the extraction of a soluble collagenous-type protein from the skin of various animals using dilute citrate buffers, and which they suggest is a soluble precursor of collagen (Plotnikova, 1947; Tustanovskii, 1947; Orekhovich, Tustanovskii, Orekhovich & Plotnikova, 1948; Chernikov, 1949; Orekhovich, 1950, 1952). The information regarding these various soluble collagens has recently been reviewed by Harkness, Marko, Muir & Neuberger (1954). These authors also report the presence of a small amount of a protein of collagen type which is extracted from skin by dilute phosphate, pH 9.0 (alkali-soluble collagen). On the basis of experiments on the feeding of labelled glycine to rabbits they conclude that this is a true precursor of collagen, whereas the metabolic role of the acid-soluble collagen de-

scribed by Orekhovich is less certain, and it is not necessarily an intermediate in the formation of all the insoluble collagen of the skin.

Harkness *et al.* (1954) determined the hydroxyproline and tyrosine content of the alkali-soluble and acid-soluble collagen, and also of the gelatin obtained from the remaining insoluble collagen. Both soluble collagens contained less tyrosine and more hydroxyproline than the insoluble collagen, and the acid-soluble had a higher hydroxyproline and tyrosine content than the alkali-soluble collagen. Similar differences have been observed by Bowes, Elliott & Moss (1953) between the hydroxyproline and tyrosine content of the acid-soluble collagen of calf skin and the adult collagen of ox hide; and between the acetic acid-soluble and insoluble fractions of tendon collagen.

A complete analysis of the acid-soluble collagen of calf skin has now been carried out in order that a more detailed comparison may be made with that of adult collagen. A preliminary account of some of these results has already been given (Bowes *et al.* 1953). The chromatographic method of Moore & Stein (1951) was used for the determination of the amino acids, and it was thought

desirable to repeat the analysis of ox-hide collagen using this method, so that direct comparison of the two proteins could be made.

In view of the apparent influence of polysaccharide on the dispersion of collagen in dilute acid solutions (Jackson, 1953) some further investigations on the sugars and amino sugars present in collagen and acid-soluble collagen have also been made.

## EXPERIMENTAL

### *Preparation of proteins*

The ox-hide collagen was from the same batch as that used in previous investigations (Bowes & Kenten, 1948*a, b*). It was originally prepared from the middle layer of a hide taken from a 2-year-old bullock immediately after flaying.

The acid-soluble collagen was prepared from the skin of a 6- to 8-month-old bull calf by the method described by Orekhovich *et al.* Immediately after flaying the skin was shaved, cut into pieces about 1 cm.<sup>2</sup> and disintegrated in a Wiley mill (A. Thomas and Co. Philadelphia). It was necessary to stop the mill at intervals and remove the macerate by hand since it would not go through even the coarsest sieve. The macerated material (1800 g.) was placed in a cotton bag and extracted as indicated in Table 1, first with 0.1M-Na<sub>2</sub>HPO<sub>4</sub>, pH 8.6, and then with 0.12M sodium citrate buffer, pH 3.62.

After the last phosphate extraction the macerate was suspended for a short time in two 3-l. portions of the citrate buffer in order to remove the phosphate buffer before going on to the citrate extractions proper. Each extraction was for a period of 24 hr., during which time the contents of the bag were agitated intermittently. All extractions were carried out between 2 and 4°. The macerated skin swelled considerably during the extractions; as much liquid as possible was squeezed out after each extraction, but relatively large amounts remained behind. After the last extraction special efforts were made to squeeze out liquid, and the relatively high protein content of this extract suggests that some liquid remaining from the earlier extractions was removed from the interstices of the protein. A small amount of the macerate (5 g.) was further extracted with successive 200-ml. portions of the citrate buffer. Samples were taken at various stages, dehydrated with acetone, and total nitrogen and hexosamine determinations were carried out.

The protein in the citrate extracts was precipitated by the addition of sufficient 30% (w/v) sodium chloride solution to bring the final concentration to 5% (w/v). The next morning the lower clear layer of liquid was withdrawn, and the top layer, containing the protein in a gelatinous form, was centrifuged. The precipitate was washed with a small amount of water and dehydrated with acetone, it having first been ascertained that this did not apparently alter the solubility of the extracted protein. The precipitated protein was further purified by redissolving in citrate buffer and dialysing against tap water.

The citrate-soluble collagen was converted into gelatin by heating a 2% (w/v) suspension in slightly acidified water to 40° for 5 min. The protein went into solution during the heating and on cooling the solution set to a gel.

### *Chemical analyses*

**Total nitrogen.** This was determined according to the method of Chibnall, Rees & Williams (1943).

**Amide-nitrogen.** The method described by Bailey (1937) and Lugg (1938*c*) was used.

**Amino-nitrogen.** The free amino-nitrogen was determined by the Van Slyke manometric procedure using a modified reaction chamber as described by Doherty & Ogg (1943).

**Amino acids.** With the exception of hydroxyproline, amino acids were determined colorimetrically with ninhydrin following separation on an ion-exchange resin (Moore & Stein, 1951). The columns used were of slightly greater diameter (1.0 cm.) than those used by Moore & Stein; this resulted in the complete separation of tyrosine and phenylalanine on the 100 cm. column, and the partial separation of hydroxylysine and histidine on the 15 cm. column used for the separation of the basic amino acids. The length of this column was increased to 20 cm. to improve this separation, and that of a third constituent eluted in the same range.

The individual peaks were identified by comparison with the behaviour of known amino acid mixtures run on similar columns and, in some instances, by paper chromatography.

In the colorimetric determination 2 ml. of ninhydrin were used. In order to keep the blank readings low it was necessary to redistill the methoxyethanol, and to recrystallize the ninhydrin. The citrate buffer used for making up the ninhydrin was shaken up with Dowex-50 resin (sodium form) to remove ammonia, and stored over this resin. Ethanol was used in place of propanol in the diluent. Several runs were made on each protein using different hydrolyses, at least two and in many cases up to four determinations being made of each amino acid.

For the majority of the amino acids the estimated error was  $\pm 3\%$  or less. In particular, for the amino acids for which differences between collagen and citrate-soluble collagen are reported, the estimated errors were: tyrosine  $\pm 4\%$ ; leucine  $\pm 2\%$ ; isoleucine  $\pm 3\%$ ; aspartic acid  $\pm 4\%$ . Rather greater errors were obtained with lysine ( $\pm 5\%$ ), valine, serine and threonine ( $\pm 6\%$ ) and methionine ( $\pm 8\%$ ). These greater errors are probably due to variable losses during hydrolysis and, in the case of valine, to the small amount present and the rather flat peak which it gives. Values for histidine and hydroxylysine were also rather variable owing to the considerable overlap of the peaks in this region. Values were chosen from the runs which gave the most satisfactory separation.

Two different methods of hydrolysis were used: conc. HCl in a sealed tube at 105° for 16 hr. and 6*N*-HCl under reflux for 24 hr. There was no evidence of any variations due to the method of hydrolysis.

**Hydroxyproline.** The method described by Neuman & Logan (1950) was used. Experiments carried out on the method of hydrolysis showed no evidence that hydrolysis under pressure (25 lb./in.<sup>2</sup>) with 6*N*-HCl for 6 hr., under reflux with 6*N*-HCl for 24 hr. or 48 hr., or in a sealed tube with conc. HCl at 105° for 16, 24 or 48 hr. gave essentially different results. In all the experiments recorded, about 200 mg. protein were hydrolysed with 10 ml. conc. HCl in a sealed tube at 105° for 16 hr. and the hydrolysate made up to 1 l. At this dilution no neutralization of the 1 or 2 ml. sample taken for the determination was necessary. In the colorimetric determination the volumes of the reagents used

by Neuman & Logan were doubled, and after development of the colour the final volume was made up to 25 ml. with distilled water. The concentration of  $\text{CuSO}_4$  was increased to 0.05M as recommended by Baker, Lampitt & Brown (1953). Readings were made on a Hilger Spekker absorptiometer using 2-cm. cells and an Ilford filter, no. 605 (max. transmission, 530–575 m $\mu$ ). Determinations were carried out in triplicate on each of two separate hydrolyses. In each determination four samples of the unknown were treated together with four standards in duplicate. Standard deviation for determinations on collagen 0.14 and for citrate-soluble collagen 0.18.

In making earlier determinations it was observed that the rate at which the  $\text{H}_2\text{O}_2$  was added was important. If the peroxide was added slowly with shaking the optical density of the hydroxyproline standards was greater than when it was added rapidly, but that of the unknowns containing the protein hydrolysate was not always similarly affected. Consequently low, and variable, values for the unknowns were often obtained. In all the determinations recorded the peroxide was added rapidly and the flasks shaken three or four times over a period of 5 min.

It was also observed that with some batches of propanol the colour was found to fade relatively rapidly, and these were discarded.

**Hexosamine.** The method of Elson & Morgan (1933) as modified by Blix (1948) was used. The reproducibility of the determination was  $\pm 5\%$  and the recovery of glucosamine

modification of the method of Lugg (1938a). Owing to the very small amount of sulphate S present in collagen, it was necessary to adapt the method to a microscale and to make corrections for sulphate present in the reagents used. Recovery of sulphate added to collagen was 93%, and the appropriate corrections were made to the values obtained for collagen alone. In view of the very small amounts present, and the larger amount of protein required, these determinations were not repeated on the citrate-soluble protein.

**Free amino groups.** The technique described by Sanger (1945), using 1-fluoro-2,4-dinitrobenzene, was used for the identification and determination of the free amino groups. Details of the methods of treatment, separation and identification have already been described (Bowes & Moss, 1953).

## RESULTS

### *Extraction of skin*

The nitrogen extracted with phosphate buffer represents about 5.5% of the total nitrogen of the macerated skin (Table 1). Protein was precipitated on the addition of ammonium sulphate to half and full saturation, and was assumed to consist of albumins and globulins, respectively. This fraction was not examined further.

Table 1. *Extraction of calf skin (1800 g.)*

For details see text.

Extracting buffer	Volume added to macerate (litres)	Volume recovered from macerate (litres)	Nitrogen content of extracts (mg./ml.)		Nitrogen as % total nitrogen of macerate	
			Before pptn.	After pptn.	Total extracted	Extracted and pptd.
0.1M- $\text{Na}_2\text{HPO}_4$ (pH 8.6)						
1	10.0	8.6	0.19	—	1.95	—
2	10.0	8.3	0.36	—	3.55	—
				Total	5.50	—
0.12M Citrate (pH 3.62)						
1	10.0	9.2	0.19	0.05	2.08	1.47
2	10.0	11.3	0.07	0.03	0.94	0.46
3	3.0	3.5	0.44	0.08	1.91	1.52
				Total	4.93	3.45
Further extractions on 5 g. macerate	(ml.)	(ml.)				
4	200	—	0.08	—	—	—
5	200	—	0.05	—	—	—
6	200	—	0.02	—	—	—

In six further extracts the nitrogen varied from 0.022 to 0.015 mg./ml.

added to collagen was high (105–110%). In subsequent experiments it was found that there was considerable interference from the other constituents of the hydrolysate (see Appendix).

The values recorded in Table 2 are uncorrected for this interference, but although too high are probably comparable with one another.

**Total and sulphate sulphur.** Total S was determined by the method of Barritt (1934) modified to deal with small amounts of sulphur. Sulphate S was determined by a

A similar proportion of the total nitrogen (4.9%) was extracted in the first three main citrate extractions. Further small amounts were extracted in the subsequent treatments of a 5 g. portion, the amounts finally becoming approximately constant after the sixth extraction at about 0.02 mg. nitrogen per ml.

On the addition of sodium chloride to the three citrate extracts between 50 and 80% of the protein

was precipitated. The protein remaining in solution probably consisted partly of albumins and globulins not completely removed by the phosphate buffer, since the addition of ammonium sulphate precipitated a further 10% of the total nitrogen of the extract. Some of the citrate-soluble collagen also probably remained in solution since the precipitation is unlikely to be complete. There is also the possibility of an additional protein fraction, for it has since been found that 10–30% of the nitrogen in citrate buffer and acetic acid solutions of tendon collagen is not precipitated by sodium chloride, even after continued extraction makes the presence of albumins and globulins from the tissue fluids extremely unlikely. This nitrogen was in the main non-dialysable, and a preliminary analysis indicated that the hydroxyproline content was negligible, the glycine, alanine and proline contents were relatively low, and the tyrosine, leucine, aspartic acid and amide contents high. It would appear, therefore, to represent a non-collagenous fraction.

The hexosamine content of the macerate was unaffected by the phosphate extractions, but was appreciably decreased by the treatments with citrate buffer (Table 2). The citrate extract contained a relatively high proportion of hexosamine

under the microscope (phase contrast), the formation of definite fibre-like particles could be seen. Some of the staining reactions of this fibrous network are listed in Table 3. The precipitate was fixed in Zenkers' fixative ( $K_2Cr_2O_7$  +  $HgCl_2$  + acetic acid). It would appear that the staining properties lie between those of collagen and reticular tissue.

The protein was soluble in dilute buffers or slightly acidified water to the extent of about 0.5 g. per 100 ml. Solubility was slightly increased in the presence of small amounts of salts and decreased with rise in pH.

On heating in water above 40° for a few minutes and cooling, the solution set to a gel, owing presumably to conversion into gelatin. The rigidity of such a gel containing the equivalent of 2 g. protein in 100 ml. 0.05M acetate buffer at pH 5.0 was determined by Mr P. R. Saunders of the British Gelatine and Glue Research Association. The determination was made at 10° after maturing for 17 hr. at the same temperature and a value of 12 000 dynes/cm.<sup>2</sup> was obtained. Gelatins of similar rigidity have been prepared in the B.G.G.R.A. Laboratories from skin and tendon, but high-grade commercial gelatins under the same conditions have lower values of the order of 9000 dynes/cm.<sup>2</sup>.

Table 2. *Nitrogen and hexosamine content of extracts and skin*

Material	Hexosamine*	Nitrogen	Hexosamine Nitrogen
(g./100 g. moisture- and ash-free protein)			
Calf skin:			
After maceration	0.41	17.66	0.023
After phosphate extraction	0.42	—	—
After citrate extraction	0.34	17.85	0.019
Precipitate from citrate extract:			
Citrate-soluble collagen	0.19	17.70	0.011
(mg./100 ml. extract)			
First citrate extract:			
Before precipitation	0.78	19.2	0.046
After precipitation	0.54	4.9	0.110

\* Uncorrected values see p. 145.

in relation to nitrogen, but only a small amount of this was carried down with the precipitated protein, the hexosamine/nitrogen ratio of the solution being increased to 0.11. These hexosamine determinations were only corrected for the colour of the hydrolysate in the absence of added Ehrlich's reagent, and not for interference of other constituents of the hydrolysate.

#### *Properties of citrate-extracted protein*

The precipitate formed on the addition of sodium chloride to the citrate extract of calf skin was fibrous in appearance and, when observed

Table 3. *Staining reactions of citrate-soluble collagen*

Stain	Reaction
Iodine	Red brown—more deeply staining than collagen
Periodic acid-Schiff	Faintly positive like collagen
Azan and Mallory's triple stain	Dense blue like collagen and reticulin
Haem alum and basic stains	Not stained, resembling collagen and reticulin
Silver	Purple to black—like reticulin rather than collagen
Toluidine blue	Not metachromatic

*Composition of collagen and procollagen*

The amino acid composition of adult ox-hide collagen, and of the citrate-soluble collagen of calf skin, both determined by the technique of Moore & Stein (1951), are given in Table 4. The composition of the same ox-hide collagen as reported in 1948 (Bowes & Kenten, 1948*a*) is also given for comparison.

agrees better with the Van Slyke value for free amino nitrogen than did the previous figure, and the new value for arginine is only slightly lower than that obtained by flavianate precipitation (14.40%) on the same material (Bowes & Kenten, 1948*b*). It was suggested at the time (Bowes & Kenten, 1948*a*) that the values for the dicarboxylic acids determined by the method of Consden, Gordon & Martin (1948) might be low, and the

Table 4. *Amino acid content of ox-hide collagen and of citrate-soluble collagen of calf skin*

	Citrate-soluble protein				Ox-hide collagen				Ox-hide collagen as reported 1948*	
	N as % protein N	g./100 g.	g. residues/ 100 g.	m-moles/g.	N as % protein N	g./100 g.	g. residues/ 100 g.	m-moles/g.	N as % protein N	g./100 g.
Total N	—	17.70	—	—	—	18.60	—	—	—	18.60
Amino N	2.65	0.49	—	0.35	2.50	0.46	—	0.33	2.50	0.46
Glycine	27.48 (4)	26.07	19.81	3.47	26.66 (2)	26.57	20.20	3.540	26.3	26.2
Alanine	8.84 (4)	9.95	7.94	1.17	8.72 (2)	10.32	8.23	1.158	8.0	9.5
Leucine	1.93 (3)	3.20	2.76	0.244	2.14 (2)	3.73	3.22	0.284	3.2	5.6
Isoleucine	0.84 (3)	1.39	1.20	0.106	1.08 (2)	1.88	1.62	0.143	—	—
Valine	1.53 (2)	2.26	1.91	0.193	1.58 (2)	2.46	2.08	0.210	2.2	3.4
Phenylalanine	0.95 (3)	1.98	1.78	0.120	1.07 (2)	2.35	1.95	0.142	1.1†	2.5
Tyrosine	0.22 (2)	0.50	0.45	0.028	0.41 (2)	0.99	0.89	0.054	0.6	1.4
Tryptophan	—	—	—	—	—	—	—	—	—	—
Serine	3.19 (4)	4.23	3.51	0.403	3.06 (4)	4.27	3.54	0.406	2.5	3.4
Threonine	1.47 (3)	2.21	1.87	0.185	1.43 (4)	2.26	1.92	0.190	1.5	2.4
Cystine	—	—	—	—	—	—	—	—	—	—
Methionine	0.41 (3)	0.78	0.68	0.052	0.49 (2)	0.97	0.85	0.065	0.4	0.8
Proline	8.95 (2)	13.02	10.98	1.131	9.43 (3)	14.42	12.16	1.252	9.9	15.1
Hydroxyproline	8.22 (6)	13.62	11.75	1.039	7.37 (6)	12.83	11.07	0.978	8.0	14.0
Arginine	15.16 (2)	8.34	7.48	0.479	14.22 (3)	8.22	7.37	0.472	15.3	8.8
Histidine	0.05 (2)	0.29	0.26	0.019	1.02 (4)	0.70	0.62	0.045	1.2	0.8
Hydroxylysine	0.88 (2)	0.90	0.80	0.065	0.93 (2)	1.00	0.89	0.062	1.2	1.3
<i>allo</i> Hydroxylysine†	0.38 (2)	0.39	0.34	0.024	0.14 (2)	0.15	0.13	0.008	—	—
Lysine	3.87 (4)	3.57	3.13	0.244	4.08 (6)	3.96	3.47	0.271	4.7	4.5
Aspartic acid	3.60 (3)	6.05	5.23	0.454	3.93 (3)	6.95	6.01	0.522	3.6	6.3
Glutamic acid	5.93 (4)	11.02	9.69	0.749	5.69 (3)	11.16	9.75	0.756	5.8	11.3
Amide	2.92 (3)	0.52	—	(0.369)	3.50 (4)	0.66	—	(0.465)	3.5	0.66
Hexosamine	—	0.01	—	—	—	0.05	—	—	—	—
Total	97.30	—	91.56	10.114§	96.97	—	95.97	10.56§	99.0	—
Average residue weight:										
By summation	—	—	90.5	—	—	90.9	—	—	—	92.6
By N-distribution	—	—	95.5	—	—	91.2	—	—	—	92.6

\* Bowes & Kenten (1948*a*).

† Tristram (1949).

‡ Not identified, but assumed to be *allo*hydroxylysine for purposes of calculation.

§ Amide excluded.

Figures in brackets indicate number of determinations made.

The present values for collagen in some instances differ slightly from those reported earlier. The values for all the basic amino acids, for tyrosine, valine, and to a lesser extent proline, are lower than the earlier values, while the values for serine, aspartic acid and alanine are rather higher. The lower value for tyrosine agrees with independent determinations made on both acid and alkaline hydrolysates by a modification of Lugg's method (Lugg, 1937, 1938*b*), viz. tyrosine N as percentage total N, 0.42. The lower value for lysine

higher value now obtained for aspartic acid substantiates this view. Valine is one of the amino acids for which the Moore & Stein method was found to give the least reproducible results and the older value is, therefore, probably to be preferred. This also applies to hydroxylysine and histidine, where overlapping of the peaks made evaluation difficult. The position of the histidine peak was determined by the addition of histidine to a hydrolysate in one run. A small amount of ninhydrin-reacting material was eluted from the 20-cm. resin

column between hydroxylysine and histidine. In view of the separation of hydroxylysine and *allo*-hydroxylysine reported by Piez (1954), it is probable that this small peak represents *allo*hydroxylysine formed by racemization during hydrolysis. A similar resolution of the hydroxylysine-histidine peak with gelatin hydrolysates is reported by Hamilton & Anderson (1954).

The values given for glycine and hydroxyproline are the first to be determined on this collagen, the previously reported values having been selected from the various determinations made by earlier workers.

The total nitrogen accounted for is 97.1% and the sum of the amino acid residues is 96.0. The average residue weight by summation is 90.9, and by calculation from the nitrogen distribution 91.2. These values are slightly lower than those previously calculated (Bowes & Kenten, 1948a).

The composition of the citrate-soluble collagen of calf skin is essentially similar to that of adult ox-hide collagen, though there are a number of small differences. In view of the lower nitrogen content compared with collagen, these differences are best compared on the basis of amino acid nitrogen as percentage total nitrogen. The amide, tyrosine and histidine contents of the citrate-soluble protein are definitely lower, and the leucine, isoleucine, and possibly the aspartic acid contents are slightly lower than in collagen, while the hydroxyproline content is higher. The ninhydrin-positive material eluted between hydroxylysine and histidine, and assumed to be *allo*hydroxylysine, is three times as great as in collagen.

The total nitrogen accounted for is 97.3% but the sum of the amino acid residues is only 91.6. This, together with the lower nitrogen content

compared with collagen, suggests the presence of some non-protein constituent.

The results of experiments on the reaction of collagen and the citrate-soluble collagen with 1-fluoro-2:4-dinitrobenzene have already been reported (Bowes & Moss, 1953). On the basis of the rather lower lysine content now found for collagen the percentage recovery of lysine as  $\epsilon$ DNP-lysine from the DNP-protein is increased from 55 to 63% and is of the same order as that found for the citrate-soluble collagen, namely 69%. Conversion of the citrate-soluble collagen into gelatin by heating to 40° for 5–10 min. resulted in an increase in the number of free  $\alpha$ -amino groups available to FDNB (Table 5). These were similar to those found in commercial gelatins (Courts, 1954), except that the proportion of glycine was much smaller. From the amounts present the number-average molecular weight was found to be 160 000.

#### *Sulphur distribution of collagen*

The sulphur distribution of the ox-hide collagen is given in Table 6. Values previously reported by Baernstein (1932) and Beach & Teague (1942) for total sulphur and ester sulphate in gelatin are rather higher, probably owing to the presence of impurities arising from contamination with hair.

An appreciable amount of the total sulphur is not accounted for; though some of this deficit may be due to cystine present as an impurity, it is probable that the value for sulphate sulphur is low owing to the difficulties of determining such small amounts.

Assuming that this sulphate sulphur is all derived from chondroitin sulphate, the amount of this polysaccharide present would be of the order 0.4%, corresponding to a galactosamine content of 0.17%.

#### DISCUSSION

The question of whether the protein extracted from skin by citrate buffers is a precursor of collagen, and represents a definite entity, or whether it represents the solution of the collagen fibres as a whole has been the subject of some discussion (Harkness, Marko, Muir & Neuberger, 1953, 1954; Neuberger, Randall & others, 1953). As far as the present experiment is concerned, the extraction of

Table 5. *Terminal residues in citrate-soluble collagen*

	Citrate-soluble collagen (m-moles/100 g. DNP-protein)	Gelatin from citrate-soluble collagen
Aspartic acid	0.06	0.14
Alanine	0.04	0.20
Glycine	—	0.14
Glutamic acid	—	0.17
Serine	—	Trace
Threonine	—	Trace

Table 6. *Sulphur distribution of gelatin and collagen*

Results are expressed as g./100 g. of moisture and ash-free protein.

	Total S	Methionine S	Cystine and cysteine S	Sulphate S	S not accounted for
Gelatin (Baernstein, 1932)	0.47	0.21	0.13	0.10	0.03
Gelatin (Beach & Teague, 1942)	0.411	0.175	0.005	0.207	0.024
Ox-hide collagen	0.30	0.18	—	0.03	0.09

calf skin with citrate buffer appears to involve, in the main, the removal of a specific soluble fraction rather than continued slow solution of the skin substance as a whole, the proportion of nitrogen extracted gradually decreasing until it reaches a low constant value. The readiness with which the citrate-soluble protein is precipitated by the addition of sodium chloride to the extract, and the fibrous nature of the precipitate formed suggests that it is more highly orientated than gelatin. On the other hand, its ready conversion into gelatin indicates that it is less heat-stable than collagen. About two-thirds of the total protein extracted by citrate buffer is precipitated by salt and is of a collagenous type (the procollagen of Orekhovich). The remaining third may partly consist of albumins and globulins, but subsequent experiments on the more exhaustive extraction of tendon indicate that a protein fraction differing from these is also present (see p.146).

The amino acid composition of ox-hide collagen as determined by the Moore & Stein (1951) technique differs slightly from that reported for the same sample in 1948 (Bowes & Kenten, 1948a). The recovery of nitrogen was not quite complete; this was probably due to overall losses of amino acids, since it was accompanied by a corresponding deficiency in the sum of the amino acid residues.

With the citrate-soluble protein the amino acids found account for 97.3% of the total nitrogen, while the sum of the weights of the amino acid residues is only 91.6 g. Thus, even if all the nitrogen unaccounted for was present as amino acids of high molecular weight, the total of the residue weights (g.) would still fall appreciably short of a hundred. This, together with the low nitrogen content compared with collagen, suggests the possible presence of about 4-5% of some constituent of low nitrogen content. No independent evidence of such a constituent has been found, however. Determination of reducing sugars and hexosamine, as well as paper chromatography and electrophoresis, indicate the presence of only very small amounts of hexosamine, galactose, glucose and mannose amounting in all to less than 0.1%, so that no appreciable amount of polysaccharide or carbohydrate is present (see Appendix).

The amino acid composition of the citrate-soluble protein differs in some respects from that reported by Chernikov (1949) and Orekhovich (1952) for procollagen. The histidine content is much lower and the sum of the proline and hydroxyproline is appreciably higher.

In view of the low nitrogen content of the citrate-soluble collagen, comparison of its composition with that of adult collagen was made on the basis of amino acid nitrogen as a percentage of the total nitrogen. It is possible that any differences ob-

served in the present investigation are due to variations in age and breed. In view, however, of the small variations in the composition of collagen from widely different sources reported by Neuman (1949) this possibility, though not completely excluded, appears to be small. The composition of the two proteins is essentially the same, but there are a few definite differences which suggest that the adult collagen is associated with a protein constituent which is relatively rich in amide nitrogen, tyrosine, histidine and, to a lesser extent, leucine, isoleucine and possibly aspartic acid, and low in hydroxyproline compared with the citrate-soluble collagen. Evidence obtained from subsequent experiments on the exhaustive extraction of tendon suggest that a protein fraction of similar composition is extracted to some extent by citrate buffers, together with the collagenous protein, and probably represents part of the protein remaining in solution after the addition of sodium chloride (see p.146). The relatively high hexosamine/nitrogen ratio of the solution after precipitation with sodium chloride suggests that this soluble protein fraction may be associated with polysaccharide.

Other workers have also found evidence for the presence of such a constituent in collagenous tissue. Eastoe & Eastoe (1954) suggest its presence in bone collagen, Consden, Glynn & Stanier (1953) in connective tissue, and Harkness *et al.* (1954) and Consden & Bird (1954) in rabbit skin. The small amounts of sugars present in collagenous tissue probably arise from this mucopolysaccharide, rather than from the collagen itself. The almost complete absence of sugars in the citrate-soluble protein would appear to substantiate this view. The sugars associated with ox-hide collagen are the same as those identified by Consden *et al.* (1953) in connective tissue and by Eastoe & Eastoe (1954) in bone collagen, though they differ in their relative proportions and are much smaller in amount (see Appendix). It is reasonable to suppose that skin with its closer fibrous structure contains less mucopolysaccharide than the looser types of collagenous tissue. It is possible that this protein-polysaccharide fraction represents the main constituent of the ground substance. It appears to be removed from collagenous tissue by alkaline treatment (Bowes *et al.* 1953; Consden & Bird, 1954; Eastoe & Eastoe, 1954), and possibly by dilute acids (Bowes *et al.* 1953; this paper, see above). This removal appears to run parallel with reduced stability of the collagen, prolonged treatment in alkaline solutions facilitating the conversion of collagen into gelatin, and treatment in dilute acetic acid favouring dispersion of the collagen. The ready conversion of the citrate-soluble collagen into gelatin also suggests that separation from the non-collagenous fraction reduces stability.

The nature of this non-collagenous protein fraction and its relationship with the citrate-soluble collagen and the insoluble collagen of adult skin is being further investigated.

### SUMMARY

1. Calf skin has been extracted with dilute citrate buffer and the soluble collagen precipitated with sodium chloride.

2. The amino acid composition of the citrate-soluble collagen as determined by the Moore & Stein (1951) technique was essentially the same as that of ox-hide collagen. The citrate-soluble collagen contained less amide N, tyrosine and histidine and rather more hydroxyproline than the ox-hide collagen. Its lysine, leucine, isoleucine and aspartic acid contents were also slightly lower.

The values obtained for some of the amino acids in ox hide differed slightly from those reported in 1948 (Bowes & Kenten, 1948a) for the same sample.

3. Reducing sugar and hexosamine determinations indicated that neither hide collagen nor the citrate-soluble collagen contained any appreciable amount of polysaccharide. The ratio of hexosamine to nitrogen in the citrate extract after precipitation of the collagenous protein suggested the presence of mucopolysaccharide.

4. Aspartic acid and alanine were detected as free terminal residues in the citrate-soluble protein. After conversion into gelatin by heating to 40° a few additional terminal residues were detected.

5. From consideration of the differences in composition between the hide collagen and the citrate-soluble protein it is suggested that the former is associated in some way with a protein constituent which is relatively rich in amide nitrogen, tyrosine, histidine and certain other amino acids, and relatively low in hydroxyproline. Other evidence for the presence of such a constituent in collagenous tissue is cited, and its possible significance with regard to the stability of collagen discussed.

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